

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

B57

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12Q 1/68, B01J 19/00	A1	(11) International Publication Number: WO 00/06770 (43) International Publication Date: 10 February 2000 (10.02.00)
(21) International Application Number: PCT/GB99/02487 (22) International Filing Date: 30 July 1999 (30.07.99) (30) Priority Data: 98306094.8 30 July 1998 (30.07.98) EP 9822670.7 16 October 1998 (16.10.98) GB (71) Applicant (for all designated States except US): SOLEXA LTD. [GB/GB]; 38 Jermyn Street, London SW1Y 6DN (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): BALASUBRAMANIAN, Shankar [GB/GB]; University of Cambridge, Dept. of Chemistry, Lensfield Road, Cambridge CB2 1EW (GB). KLENERMAN, David [GB/GB]; University of Cambridge, Dept. of Chemistry, Lensfield Road, Cambridge CB2 1EW (GB). (74) Agent: GILL JENNINGS & EVERY; Broadgate House, 7 Eldon Street, London EC2M 7LH (GB).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: ARRAYED BIOMOLECULES AND THEIR USE IN SEQUENCING		
(57) Abstract According to the present invention, a device comprising an array of molecules immobilised on a solid surface is disclosed, wherein the array has a surface density which allows each molecule to be individually resolved, e.g. by optical microscopy. Therefore, the arrays of the present invention consist of single molecules that are more spatially distinct than the arrays of the prior art.		

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

ARRAYED BIOMOLECULES AND THEIR USE IN SEQUENCING

Field of the Invention

This invention relates to fabricated arrays of molecules, and to their analytical applications. In particular, this invention relates to the use of fabricated arrays in methods for obtaining genetic sequence information.

Background of the Invention

Advances in the study of molecules have been led, in part, by improvement in technologies used to characterise the molecules or their biological reactions. In particular, the study of nucleic acids, DNA and RNA, has benefited from developing technologies used for sequence analysis and the study of hybridisation events.

An example of the technologies that have improved the study of nucleic acids, is the development of fabricated arrays of immobilised nucleic acids. These arrays typically consist of a high-density matrix of polynucleotides immobilised onto a solid support material. Fodor *et al.*, Trends in Biotechnology (1994) 12:19-26, describes ways of assembling the nucleic acid arrays using a chemically sensitised glass surface protected by a mask, but exposed at defined areas to allow attachment of suitably modified nucleotides. Typically, these arrays may be described as "many molecule" arrays, as distinct regions are formed on the solid support comprising a high density of one specific type of polynucleotide.

An alternative approach is described by Schena *et al.*, Science (1995) 270:467-470, where samples of DNA are positioned at predetermined sites on a glass microscope slide by robotic micropipetting techniques. The DNA is attached to the glass surface along its entire length by non-covalent electrostatic interactions. However, although hybridisation with complementary DNA sequences can occur, this approach may not permit the DNA to be freely available for interacting with other components such as polymerase enzymes, DNA-binding proteins etc.

The arrays are usually provided to study hybridisation events, to determine the sequence of DNA (Mirzabekov, Trends. in Biotechnology (1994) 12:27-32) or to detect mutations in a particular DNA sample. Many of these hybridisation events are detected using fluorescent labels attached to nucleotides, the labels being detected using a sensitive fluorescent detector, e.g. a charge-coupled detector (CCD). The major disadvantages of these methods are that it is not possible to sequence long stretches of DNA and that

repeat sequences can lead to ambiguity in the results. These problems are recognised in Automation Technologies for Genome Characterisation, Wiley-Interscience (1997), ed. T. J. Beugelsdijk, Chapter 10: 205-225.

In addition, the use of high-density arrays in a multi-step analysis procedure can
5 lead to problems with phasing. Phasing problems result from a loss in the synchronisation of a reaction step occurring on different molecules of the array. If some of the arrayed molecules fail to undergo a step in the procedure, subsequent results obtained for these molecules will no longer be instep with results obtained for the other arrayed molecules. The proportion of molecules out of phase will increase through
10 successive steps and consequently the results detected will become ambiguous. This problem is recognised in the sequencing procedure described in US-A-5302509.

An alternative sequencing approach is disclosed in EP-A-0381693, which comprises hybridising a fluorescently-labelled strand of DNA to a target DNA sample suspended in a flowing sample stream, and then using an exonuclease to cleave
15 repeatedly the end base from the hybridised DNA. The cleaved bases are detected in sequential passage through a detector, allowing reconstruction of the base sequence of the DNA. Each of the different nucleotides has a distinct fluorescent label attached, which is detected by laser-induced fluorescence. This is a complex method, primarily because it is difficult to ensure that every nucleotide of the DNA strand is labelled and
20 that this has been achieved with high fidelity to the original sequence.

Summary of the Invention

The present invention is based in part at least on the realisation that molecule arrays can be produced with sufficient separation between the molecules to provide distinct optical resolution. The arrays may be formed by simply immobilising a mixture
25 of molecules to a solid surface in such a way that provides sufficient separation between the molecules to allow each molecule to be resolved optically.

According to the present invention, a device comprises an array of molecules capable of interrogation and immobilised on a solid surface, wherein the array has a surface density which allows each molecule to be individually resolved, e.g. by optical
30 microscopy, and wherein each molecule is immobilised at one or more points, by specific interaction with the surface, other than at that part of each molecule that can be interrogated. Therefore, the arrays of the present invention comprise what are effectively

single molecules that are more spatially distinct than the arrays of the prior art. This has many important benefits for the study of the molecules and their interaction with other biological molecules. In particular, fluorescence events occurring to each molecule can be detected using an optical microscope linked to a sensitive detector, resulting in a distinct signal for each molecule.

When used in a multi-step analysis of a population of single molecules there is a removal of the phasing problems that are encountered using high density arrays of the prior art. Therefore, the novel arrays also permit a massively parallel approach to monitoring fluorescent or other events on the molecules. Such massively parallel data acquisition makes the arrays extremely useful in a wide range of analysis procedures which involve the screening/characterising of heterogeneous mixtures of molecules. The arrays can be used to characterise a particular synthetic chemical or biological moiety, for example in screening procedures to identify particular molecules produced in combinatorial synthesis reactions.

The arrayed molecules may be immobilised on a solid support via microspheres. A microsphere can be visualised easily, allowing it to be positioned within a distinct optically resolvable region of a microscope prior to carrying out further analysis procedures.

The arrays may be used in many different analysis procedures or characterisation studies. In one embodiment, the molecules are polynucleotides, and the arrays permit sequence determinations to be carried out.

Generally, any sequencing method can be used which makes use of fluorescent or other labels to identify particular nucleotides or sequences of nucleotides. A preferred method comprises the repeated steps of: reacting an immobilised target polynucleotide with a primer, a polymerase and the different nucleoside triphosphates under conditions sufficient for the polymerase reaction to proceed, wherein each nucleoside triphosphate is conjugated at its 3' position to a different fluorescent label, determining which label (and thus which nucleotide) has undergone the polymerase reaction, and removing the label. Because the method utilises the arrays of the present invention, each incorporated nucleotide can be unambiguously determined by fluorescent measurements, and additionally the method can be used to detect many thousands of reactions at the same time with no phasing problems.

Alternatively, the arrays may be used in genotyping procedures (as disclosed in Shalon *et al*, Genome Research (1996) 639-645), to provide a genetic "bar code" for an organism, mapping studies and mRNA-based expression monitoring (as disclosed in Wodicka *et al*, Nat. Biotechnol. (1997) 15:1359). The arrays may also be used as a
5 sensor, in the manner disclosed in Analytical Chemistry (1998) 70:1242-1248.

According to a further aspect of the invention, a method comprises contacting, under suitable conditions, an immobilised array of polynucleotides according to the present invention, of predetermined sequence, with a plurality of target molecules capable of binding to the arrayed polynucleotides, and detecting a binding event, thereby
10 determining the position of a bound molecule on the array. This method permits identification of molecules synthesised by the combinatorial chemistry reactions and incorporating, for example, a polynucleotide identifier tag.

A further method comprises the steps of contacting an array of polynucleotides according to the invention with a plurality of detectably-labelled fragments of an
15 organism's genomic DNA, under hybridising conditions, and detecting hybridisation events. The organism may be mammalian, in particular human, or alternatively the organism may be bacterial or viral. This method allows genotyping analysis to be carried out.

An array of the invention may be used to generate a spatially addressable array
20 of single polynucleotide molecules. This is the simple consequence of sequencing the array. Particular advantages of such a spatially addressable array include the following:

- 1) Polynucleotide molecules on the array may act as identifier tags and may only need to be 10-20 bases long, and the efficiency required in the sequencing steps may only need to be better than 95%.
- 25 2) The arrays may be reusable for screening once created and sequenced. All possible sequences can be produced in a very simple way, e.g. compared to a high density DNA chip made using photolithography.

Description of the Drawings

Figure 1 is a schematic representation of apparatus that may be used to image
30 arrays of the present invention;

Figure 2 illustrates the immobilisation of a polynucleotide to a solid surface via a microsphere;

Figure 3 shows a fluorescence time profile from a single fluorophore-labelled oligonucleotide, with excitation at 514nm and detection at 600nm;

Figure 4 shows fluorescently labelled single molecule DNA covalently attached to a solid surface; and

5 Figure 5 shows images of surface bound oligonucleotides hybridised with the complementary sequence.

Description of the Invention

According to the present invention, the single molecules immobilised onto the surface of a solid support must be capable of being individually resolved, e.g. by optical
10 means. This means that, within the resolvable area of the particular imaging device used, there must be one or more distinct images each representing one molecule. Typically, the molecules of the array are resolved using a single molecule fluorescence microscope equipped with a sensitive detector, e.g. a charge-coupled detector (CCD), each molecule of the array being analysed simultaneously.

15 The molecules of the array may be any biomolecule including peptides and polypeptides, but in particular DNA and RNA and nucleic acid mimics, e.g. PNA and 2'-O-methRNA. However, other organic molecules may also be used. The molecules are formed on the array to allow interaction with other "cognate" molecules. It is therefore important to immobilise the molecules so that the portion of the molecule not
20 used to immobilise the molecule, is capable of being interrogated by a cognate. In some applications all the molecules in the single array will be the same, and may be used to interrogate molecules that are largely distinct. In other applications, the molecules on the array will primarily be distinct, e.g. more than 50%, preferably more than 70% of the molecules will be different to that of the other molecules.

25 The arrays of the present invention are single molecule arrays. The term "single molecule" is used herein to refer to one molecule that is visualised separately from neighbouring molecules (whether or not each molecule is of the same or different type).

The term "individually resolved" is used herein to specify that, when visualised, it is possible to distinguish one molecule on the array from its neighbouring molecules.
30 Visualisation is effected by the use of reporter labels, e.g. fluorophores, the signal of which is individually resolved.

The term "cognate molecule" is used herein to refer to any molecule capable of interacting, or interrogating, the arrayed molecule. The cognate may be a molecule that binds specifically to the arrayed molecule, for example a complementary polynucleotide, in a hybridisation reaction. Alternatively, the cognate may associate non-specifically with the arrayed molecule, for example a polymerase enzyme which associates with an arrayed polynucleotide in the process of synthesising a complementary strand.

The term "interrogate" is used herein to refer to any interaction of the arrayed molecule with any other molecule. The interaction may be covalent or non-covalent.

The terms "arrayed polynucleotides" and "polynucleotide arrays" are used herein to define an array of single molecules that are characterised by comprising a polynucleotide molecule. The term is intended to include the attachment of other molecules to a solid surface, the molecules having a polynucleotide attached that can be further interrogated. For example, the arrays may comprise protein molecules immobilised on a solid surface, the protein molecules being conjugated with or otherwise bound to a short polynucleotide molecule may be interrogated, to address the array.

The extent of separation between the individual molecules on the array will be determined, in part, by the particular technique used to resolve the individual molecule. Apparatus used to image molecular arrays are known to those skilled in the art. For example, a confocal scanning microscope may be used to scan the surface of the array with a laser to image directly a fluorophore incorporated on the individual molecule by fluorescence, as shown in Figure 1, where (1) represents a detector, (2) a bandpass filter, (3) a pinhole, (4) a mirror, (5) a laser beam, (6) a dichroic mirror, (7) an objective, (8) a glass coverslip and (9) a sample under study. Alternatively, a sensitive 2-D detector, such as a charge-coupled detector, can be used to provide a 2-D image representing the individual molecules on the array. In this example, resolving single molecules on the array is possible if the molecules are separated by a distance of approximately at least 250nm x 250nm, preferably at least 300nm x 300nm and more preferably by at least 350nm x 350nm.

However, other techniques such as scanning near-field optical microscopy (SNOM) are available which are capable of smaller optical resolutions, thereby permitting "more dense" arrays to be used. For example, using SNOM, the molecules may be separated by a distance of less than 100nm, e.g. 10nm x 10nm. For a description of

scanning near-field optical microscopy, see Moyer *et al.*, Laser Focus World (1993) 29(10).

Additionally, the techniques of scanning tunnelling microscopy (Binnig *et al.*, Helvetica Physica Acta (1982) 55:726-735) and atomic force microscopy (Hanswa *et al.*,
5 Annu. Rev. Biophys. Biomol. Struct. (1994) 23:115-139) are suitable for imaging the arrays of the present invention. Other devices which do not rely on microscopy may also be used, provided that they are capable of imaging within discrete areas on a solid support.

Single molecules may be arrayed by immobilisation to the surface of a solid
10 support. This may be carried out by any known technique, provided that suitable conditions are used to ensure adequate separation of the molecules. Generally the array is produced by dispensing small volumes of a sample containing a mixture of molecules onto a suitably prepared solid surface, or by applying a dilute solution to the solid surface to generate a random array. In this manner, a mixture of different molecules may be
15 arrayed by simple means. The formation of the single molecule array then permits identification of each arrayed molecule to be carried out.

It is important to prepare the solid support under conditions which minimise or avoid the presence of contaminants. The solid support must be cleaned thoroughly, preferably with a suitable detergent, e.g. Decon-90, to remove dust and other
20 contaminants.

Immobilisation may be by specific covalent or non-covalent interactions. If the molecule is a polynucleotide, immobilisation will preferably be at either the 5' or 3' position, so that the polynucleotide is attached to the solid support at one end only. However, the polynucleotide may be attached to the solid support at any position along
25 its length, the attachment acting to tether the polynucleotide to the solid support. The immobilised polynucleotide is then able to undergo interactions with other molecules or cognates at positions distant from the solid support. Typically the interaction will be such that it is possible to remove any molecules bound to the solid support through non-specific interactions, e.g. by washing. Immobilisation in this manner results in well
30 separated single molecules. The advantage of this is that it prevents interaction between neighbouring molecules on the array, which may hinder interrogation of the array.

In one embodiment of the invention, the surface of a solid support is first coated with streptavidin or avidin, and then a dilute solution of a biotinylated molecule is added at discrete sites on the surface using, for example, a nanolitre dispenser to deliver one molecule on average to each site. If the molecule is a polynucleotide, then
5 immobilisation may be via hybridisation to a complementary nucleic acid molecule previously attached to a solid support. For example, the surface of a solid support may be first coated with a primer polynucleotide at discrete sites on the surface. Single-stranded polynucleotides are then brought into contact with the arrayed primers under hybridising conditions and allowed to "self-sort" onto the array. In this way, the arrays
10 may be used to separate the desired polynucleotides from a heterogeneous sample of polynucleotides.

Alternatively, the arrayed primers may be composed of double-stranded polynucleotides with a single-stranded overhang ("sticky-ends"). Hybridisation with target polynucleotides is then allowed to occur and a DNA ligase used to covalently link
15 the target DNA to the primer. The second DNA strand can then be removed under melting conditions to leave an arrayed polynucleotide.

In a preferred embodiment of the invention, the solid surface is coated with an epoxide and the molecules are coupled via an amine linkage. It is also preferable to avoid or reduce salt present in the solution containing the molecule to be arrayed. Reducing
20 the salt concentration minimises the possibility of the molecules aggregating in the solution, which may affect the positioning on the array.

In an embodiment of the invention, the target molecules are immobilised onto non-fluorescent streptavidin or avidin-functionalised polystyrene latex microspheres, as shown in Fig. 2 where (1) represents the microsphere, (2) a streptavidin molecule (3) a
25 biotin molecule and (4) a fluorescently labelled polynucleotide. The microspheres are immobilised in turn onto a solid support to fix the target sample for microscope analysis. Alternative microspheres suitable for use in the present invention are well known in the art.

The single molecule arrays have many applications in methods which rely on the
30 detection of biological or chemical interactions with arrayed molecules. For example, the arrays may be used to determine the properties or identities of cognate molecules.

Typically, interaction of biological or chemical molecules with the arrays are carried out in solution.

In particular, the arrays may be used in conventional assays which rely on the detection of fluorescent labels to obtain information on the arrayed molecules. The arrays are particularly suitable for use in multi-step assays where the loss of synchronisation in the steps was previously regarded as a limitation to the use of arrays. When the arrays are composed of polynucleotides they may be used in conventional techniques for obtaining genetic sequence information. Many of these techniques rely on the stepwise identification of suitably labelled nucleotides, referred to in US-A-5634413 as "single base" sequencing methods.

In an embodiment of the invention, the sequence of a target polynucleotide is determined in a similar manner to that described in US-A-5634413, by detecting the incorporation of nucleotides into the nascent strand through the detection of a fluorescent label attached to the incorporated nucleotide. The target polynucleotide is primed with a suitable primer, and the nascent chain is extended in a stepwise manner by the polymerase reaction. Each of the different nucleotides (A, T, G and C) incorporates a unique fluorophore at the 3' position which acts as a blocking group to prevent uncontrolled polymerisation. The polymerase enzyme incorporates a nucleotide into the nascent chain complementary to the target, and the blocking group prevents further incorporation of nucleotides. The array surface is then cleared of unincorporated nucleotides and each incorporated nucleotide is "read" optically by a charge-coupled detector using laser excitation and filters. The 3' -blocking group^f is then removed (deprotected), to expose the nascent chain for further nucleotide incorporation.

Because the array consists of distinct optically resolvable polynucleotides, each target polynucleotide will generate a series of distinct signals as the fluorescent events are detected. Details of the full sequence are then determined.

The number of cycles that can be achieved is governed principally by the yield of the deprotection cycle. If deprotection fails in one cycle, it is possible that later deprotection and continued incorporation of nucleotides can be detected during the next cycle. Because the sequencing is performed at the single molecule level, the sequencing can be carried out on different polynucleotide sequences at one time without the

necessity for separation of the different sample fragments prior to sequencing. This sequencing also avoids the phasing problems associated with prior art methods.

Deprotection may be carried out by chemical, photochemical or enzymatic reactions.

5 A similar, and equally applicable, sequencing method is disclosed in EP-A-0640146.

Other suitable sequencing procedures will be apparent to the skilled person. In particular, the sequencing method may rely on the degradation of the arrayed polynucleotides, the degradation products being characterised to determine the sequence.

10 An example of a suitable degradation technique is disclosed in WO-A- 95/20053, whereby bases on a polynucleotide are removed sequentially, a predetermined number at a time, through the use of labelled adaptors specific for the bases, and a defined exonuclease cleavage.

However, a consequence of sequencing using non-destructive methods is that it is possible to form a spatially addressable array for further characterisation studies, and therefore non-destructive sequencing may be preferred. In this context, term "spatially addressable" is used herein to describe how different molecules may be identified on the basis of their position on an array.

Once sequenced, the spatially addressed arrays may be used in a variety of procedures which require the characterisation of individual molecules from heterogeneous populations.

One application is to use the arrays to characterise products synthesised in combinatorial chemistry reactions. During combinatorial synthesis reactions, it is usual for a tag or label to be incorporated onto a beaded support or reaction product for the subsequent characterisation of the product. This is adapted in the present invention by using polynucleotide molecules as the tags, each polynucleotide being specific for a particular product, and using the tags to hybridise onto a spatially addressed array. Because the sequence of each arrayed polynucleotide has been determined previously, the detection of an hybridisation event on the array reveals the sequence of the complementary tag on the product. Having identified the tag, it is then possible to confirm which product this relates to. The complete process is therefore quick and

simple, and the arrays may be reused for high through-put screening. Detection may be carried out by attaching a suitable label to the product, e.g. a fluorophore.

Combinatorial chemistry reactions may be used to synthesise a diverse range of different molecules, each of which may be identified using the addressed arrays of the present invention. For example, combinatorial chemistry may be used to produce therapeutic proteins or peptides that can be bound to the arrays to produce an addressed array of target proteins. The targets may then be screened for activity, and those proteins exhibiting activity may be identified by their position on the array as outlined above.

Similar principles apply to other products of combinatorial chemistry, for example the synthesis of non-polymeric molecules of M.wt.<1000. Methods for generating peptides/proteins by combinatorial methods are disclosed in US-A-5643768 and US-A-5658754. Split-and-mix approaches may also be used, as described in Nielsen *et al.*, J. Am. Chem. Soc. (1993) 115:9812-9813.

In an alternative approach, the products of the combinatorial chemistry reactions may comprise a second polynucleotide tag not involved in the hybridisation to the array. After formation by hybridisation, the array may be subjected to repeated polynucleotide sequencing to identify the second tag which remains free. The sequencing may be carried out as described previously.

Therefore, in this application, it is the tag that provides the spatial address on the array. The tag may then be removed from the product by, for example, a cleavable linker, to leave an untagged spatially addressed array.

A further application is to display proteins via an immobilised polysome containing trapped polynucleotides and protein in a complex, as described in US 5643768 and US 5658754.

In a separate embodiment of the invention, the arrays may be used to characterise an organism. For example, an organism's genomic DNA may be screened using the arrays, to reveal discrete hybridisation patterns that are unique to an individual. This embodiment may therefore be likened to a "bar code" for each organism. The organism's genomic DNA may be first fragmented and detectably-labelled, for example with a fluorophore. The fragmented DNA is then applied to the array under hybridising conditions and any hybridisation events monitored.

Alternatively, hybridisation may be detected using an in-built fluorescence based detection system in the arrayed molecule, for example using the "molecular beacons" described in Nature Biotechnology (1996) 14:303-308.

5 It is possible to design the arrays so that the hybridisation pattern generated is unique to the organism and so could be used to provide valuable information on the genetic character of an individual. This may have many useful applications in forensic science. Alternatively, the methods may be carried out for the detection of mutations or allelic variants within the genomic DNA of an organism.

10 For genotyping, it is desirable to identify if a particular sequence is present in the genome. The smallest possible unique oligomer is a 16-mer (assuming randomness of the genome sequence), i.e. statistically there is a probability of any given 16-base sequence occurring only once in the human genome (which has 3×10^9 bases). There are c. 4×10^9 possible 16-mers which would fit within a region of 2 cm x 2 cm (assuming a single copy at a density of 1 molecule per 250 nm x 250 nm square). It is therefore
15 necessary to determine only if a particular 16-mer is present or not, and so quantitative measurements are unnecessary. Identifying a mutation in a particular region and what the mutation is can be carried out using the 16-mer library. Mapping back onto the human genome would be possible using published data and would not be a problem once the entire genome has been determined. There is built-in self-check, by looking at the
20 hybridisation to particular 16-mers so that if there is a single point mutation, this will show up in 16 different 16-mers, identifying a region of 32 bases in the genome (the mutation would occur at the top of one 16-mer and then at the second base in a related 16-mer etc). Thus, a single point mutation would result in 16 of the 16-mers not showing hybridisation and a new set of 16 showing hybridisation plus the same thing for
25 the complementary strand. In summary, considering both strands of DNA, a single point mutation would result in 32 of the 16-mers not showing hybridisation and 32 new 16-mers showing hybridisation, i.e. quite large changes on the hybridisation pattern to the array.

By way of example, a sample of human genomic DNA may be restriction-digested
30 to generate short fragments, then labelled using a fluorescently-labelled monomer and a DNA polymerase or a terminal transferase enzyme. This produces short lengths of sample DNA with a fluorophore at one end. The melted fragments may then be exposed

to the array and the pixels where hybridisation occurs or not would be identified. This produces a genetic bar code for the individual with (if oligonucleotides of length 16 were used) $c.4 \times 10^9$ binary coding elements. This would uniquely define a person's genotype for pharmagenomic applications. Since the arrays should be reusable, the same process
5 could be repeated on a different individual.

Viral and bacterial organisms may also be studied, and screening nucleic acid samples may reveal pathogens present in a disease, or identify microorganisms in analytical techniques. For example, pathogenic or other bacteria may be identified using a series of single molecule DNA chips produced from different strains of bacteria. Again,
10 these chips are simple to make and reusable.

In a further example, double-stranded arrays may be used to screen protein libraries for binding, using fluorescently labelled proteins. This may determine proteins that bind to a particular DNA sequence, i.e. proteins that control transcription. Once the short sequence that the protein binds to has been determined, it may be made and affinity
15 purification used to isolate and identify the protein. Such a method could find all the transcription-controlling proteins. One such method is disclosed in Nature Biotechnology (1999) 17:p573-577.

Another use is in expression monitoring. For this, a label is required for each gene. There are $c.100,000$ genes in the human genome. There are 262,144 possible 9-
20 mers, so this is the minimum length of oligomer needed to have a unique tag for each gene. This 9-mer label needs to be at a specific point in the DNA and the best point is probably immediately after the poly-A tail in the mRNA (i.e. a 9-mer linked to a poly-T guide sequence). Multiple copies of these 9-mers should be present, to permit quantitation of gene expression. 100 copies would allow determination of relative
25 expression from 1-100%. 10,000 copies would allow determination of relative gene expression from .01-100%. 10,000 copies of 262,144 9-mers would fit inside 1 cm x 1 cm at close to maximum density.

The use of nanovials in conjunction with any of the above methods may allow a molecule to be cleaved from the surface, yet retain its spatial integrity. This permits the
30 generation of spatially addressable arrays of single molecules in free solution, which may have advantages where the surface attachment impedes the analysis (e.g. drug screening). A nanovial is a small cavity in a flat glass surface, e.g. approx 20 μm in diameter and 10

µm deep. They can be placed every 50 µm, and so the array would be less dense than a surface-attached array; however, this could be compensated for by appropriate adjustment in the imaging optics.

5 The following Examples illustrate the invention, with reference to the accompanying drawings.

Example 1

10 The microscope set-up used in the following Example was based on a modified confocal fluorescence system using a photon detector as shown in Figure 1. Briefly, a narrow, spatially filtered laser beam (CW Argon Ion Laser Technology RPC50) was passed through an acousto-optic modulator (AOM) (A.A Opto-Electronic) which acts as a fast optical switch. The acousto-optic modulator was switched on and the laser beam was directed through an oil emersion objective (100 X, NA = 1.3) of an inverted optical microscope (Nikon Diaphot 200) by a dichroic beam splitter (540DRLP02 or 505DRLP02, Omega Optics Inc.). The objective focuses the light to a diffraction-limited spot on the target sample immobilised on a thin glass coverslip. Fluorescence from the sample was collected by the same objective, passed through the dichroic beam splitter and directed through a 50 µm pinhole (Newport Corp.) placed in the image plane of the microscope observation port. The pinhole rejects light emerging from the sample which is out of the plane of the laser focus. The transmitted fluorescence was separated spectrally by a dichroic beam splitter into red and green components which was filtered to remove residual laser scatter. The remaining fluorescence components were then focused onto separate single photon avalanche diode detectors and the signals recorded onto a multichannel scalar (MCS) (MCS-Plus, EG & G Ortec) with time resolutions in the 1 to 10 ms range.

25 The target sample was a 5'-biotin-modified 13-mer primer oligonucleotide prepared using conventional phosphoramidite chemistry, and having SEQ ID No. 1 (see listing, below). The oligonucleotide was post-synthetically modified by reaction of the uridine base with the succinimide ester of tetramethylrhodamine (TMR).

30 Glass coverslips were prepared by cleaning with acetone and drying under nitrogen. A 50 µl aliquot of biotin-BSA (Sigma) redissolved in PBS buffer (0.01 M, pH 7.4) at 1 mg/ml concentration was deposited on the clean coverslip and incubated for 8 hours at 30°C. Excess biotin-BSA was removed by washing 5 times with MilliQ water

and drying under nitrogen. Non-fluorescent streptavidin functionalised polystyrene latex microspheres of diameter 500nm (Polysciences Inc.) were diluted in 100 mM NaCl to 0.1 solids and deposited as a 1 μ l drop on the biotinylated coverslip surface. The spheres were allowed to dry for one hour and unbound beads removed by washing 5 times with
5 MilliQ water. This procedure resulted in a surface coverage of approximately 1 sphere/100 μ m x 100 μ m.

The non-fluorescent microspheres were found to have a broad residual fluorescence at excitation wavelength 514nm, probably arising from small quantities of photoactive constituents used in the colloidal preparation of the microspheres. The
10 microspheres were therefore photobleached by treating the prepared coverslip in a laser beam of a frequency doubled (532nm) Nd:YAG pulsed dye laser, for 1 hour.

The biotinylated 13-TMR ssDNA was coupled to the streptavidin functionalised microspheres by incubating a 50 μ l sample of 0.1 pM DNA (diluted in 100 mM NaCl, 100 mM Tris) deposited over the microspheres. Unbound DNA was removed by
15 washing the coverslip surface 5 times with MilliQ water.

Low light level illumination from the microscope condenser was used to position visually a microsphere at 10x magnification so that when the laser was switched on the sphere was located in the centre of the diffraction limited focus. The condenser was then turned off and the light path switched to the fluorescence detection port. The MCS was
20 initiated and the fluorescence omitted from the latex sphere recorded on one or both channels. The sample was excited at 514nm and detection was made on the 600nm channel.

Figure 3 shows clearly that the fluorescence is switched on as the laser is deflected into the microscope by the AOM, 0.5 seconds after the start of a scan. The
25 intensity of the fluorescence remains relatively constant for a short period of time (100 ms-3s) and disappears in a single step process. The results show that single molecule detection is occurring. This single step photobleaching is unambiguous evidence that the fluorescence is from a single molecule.

Example 2

30 This Example illustrates the preparation of single molecule arrays by direct covalent attachment to glass followed by a demonstration of hybridisation to the array.

Covalently modified slides were prepared as follows. Spectrosil-2000 slides (TSL, UK) were rinsed in milli-Q to remove any dust and placed wet in a bottle containing neat Decon-90 and left for 12 h at room temperature. The slides were rinsed with milli-Q and placed in a bottle containing a solution of 1.5% glycidoxypolytrimethoxy-silane in milli-Q and magnetically stirred for 4 h at room temperature rinsed with milli-Q and dried under N₂ to liberate an epoxide coated surface.

The DNA used was that shown in SEQ ID No. 2 (see sequence listing below), where n represents a 5-methyl cytosine (Cy5) with a TMR group coupled via a linker to the n4 position.

10 A sample of this (5 µl, 450 pM) was applied as a solution in neat milli-Q.

The DNA reaction was left for 12 h at room temperature in a humid atmosphere to couple to the epoxide surface. The slide was then rinsed with milli-Q and dried under N₂.

15 The prepared slides can be stored wrapped in foil in a desiccator for at least a week without any noticeable contamination or loss of bound material. Control DNA of the same sequences and fluorophore but without the 5'-amino group shows little stable coverage when applied at the same concentration.

The TMR labelled slides were then treated with a solution of complementary DNA (SEQ ID No. 3) (5µM, 10µl) in 100mM PBS. The complementary DNA has the sequence shown in SEQ ID No. 3, where n represents a methylcytosine group.

20 After 1 hour at room temperature the slides were cooled to 4°C and left for 24 hours. Finally, the slides were washed in PBS (100mM, 1mL) and dried under N₂.

25 A chamber was constructed on the slide by sealing a coverslip (No. 0, 22x22mm, Chance Propper Ltd, UK) over the sample area on two sides only with prehardened microscope mounting medium (Eukitt, O. Kindler GmbH & Co., Freiburg, Germany) whilst maintaining a gap of less than 200µm between slide and coverslip. The chamber was flushed 3x with 100µl PBS (100mM NaCl) and allowed to stabilise for 5 minutes before analysing on a fluorescence microscope.

30 The slide was inverted so that the chamber coverslip contacted the objective lens of an inverted microscope (Nikon TE200) via an immersion oil interface. A 60° fused silica dispersion prism was optically coupled to the back of the slide through a thin film of glycerol. Laser light was directed at the prism such that at the glass/sample interface

it subtends an angle of approximately 68° to the normal of the slide and subsequently undergoes Total Internal Reflection (TIR). The critical angle for glass/water interface is 66° .

Fluorescence from single molecules of DNA-TMR or DNA-Cy5 produced by
5 excitation with the surface specific evanescent wave following TIR is collected by the objective lens of the microscope and imaged onto an Intensified Charge Coupled Device (ICCD) camera (Pentamax, Princeton Instruments, NJ). Two images were recorded using a combination of 1) 532nm excitation (frequency doubled solid state Nd:YAG, Antares, Coherent) with a 580nm fluorescence (580DF30, Omega Optics, USA) filter
10 for TMR and 2) 630nm excitation (Nd:YAG pumped dye laser, Coherent 700) with a 670nm filter (670DF40, Omega Optics, USA) for Cy5. Images were recorded with an exposure time of 500ms at the maximum gain of 10 on the ICCD. Laser powers incident at the prism were 50mW and 40mW at 532nm and 630nm respectively. A third image was taken with 532nm excitation and detection at 670nm to determine the level of cross-talk from TMR on the Cy5 channel.
15

Single molecules were identified by single points of fluorescence with average intensities greater than 3x that of the background. Fluorescence from a single molecule is confined to a few pixels, typically a 3x3 matrix at 100x magnification, and has a narrow Gaussian-like intensity profile. Single molecule fluorescence is also characterised by a
20 one-step photobleaching process in the time course of the intensity and was used to distinguish single molecules from pixel regions containing two or more molecules, which exhibited multi-step processes. Figures 4a and 4b show $60 \times 60 \mu\text{m}^2$ fluorescence images from covalently modified slides with DNA-TMR starting concentrations of 45pM and 450pM. Figure 4c shows a control slide which was treated as above but with DNA-TMR
25 lacking the 5' amino modification.

To count molecules a threshold for fluorescence intensities is first set to exclude background noise. For a control sample the background is essentially the thermal noise of the ICCD measured to be 76 counts with a standard deviation of only 6 counts. A threshold is arbitrarily chosen as a linear combination of the background, the average
30 counts over an image and the standard deviation over an image. In general, the latter two quantities provide a measure of the number of pixels and range of intensities above background. This method gives rise to threshold levels which are at least 12 standard

deviations above the background with a probability of less than 1 in 144 pixels contributing from noise. By defining a single molecule fluorescent point as being at least a 2x2 matrix of pixels and no larger than a 7x7, the probability of a single background pixel contributing to the counting is eliminated and clusters are ignored.

5 In this manner, the surface density of single molecules of DNA-TMR is measured at 2.9×10^6 molecules/cm² (238 molecules in Figure 4a) and 5.8×10^6 molecules/cm² (469 molecules in Figure 4b) at 45pM and 450pM DNA-TMR coupling concentrations. The density is clearly not directly proportional to DNA concentration but will be some function of the concentration, the volume of sample applied, the area covered by the
10 sample and the incubation time. The percentage of non-specifically bound DNA-TMR and impurities contribute of the order of 3-9% per image (8 non-specifically bound molecules in Figure 4c). Analysis of the photobleaching profiles shows only 6% of fluorescence points contain more than 1 molecule.

 Hybridisation was identified by the co-localisation of discrete points of
15 fluorescence from single molecules of TMR and Cy-5 following the superposition of two images. Figures 5a and 5b show images of surface bound 20-mer labelled with TMR and the complementary 20-mer labelled with Cy-5 deposited from solution. Figure 5d shows those fluorescent points that are co-localised on the two former images. The degree of hybridisation was estimated to be 7% of the surface-bound DNA (10 co-localised points
20 in 141 points from Figures 5d and 5a respectively). The percentage of hybridised DNA is estimated to be 37% of all surface-adsorbed DNA-Cy5 (10 co-localised points in 27 points from Figures 5d and 5b respectively). Single molecules were counted by matching size and intensity of fluorescent points to threshold criteria which separate single molecules from background noise and cosmic rays. Figure 5d shows the level of cross-
25 talk from TMR on the Cy5 channel which is to be 2% as determined by counting only those fluorescent points which fall within the criteria for determining the TMR single molecule fluorescence (2 fluorescence points in 141 from Figures 5c and 5a respectively).

 This Example demonstrates that single molecule arrays can be formed, and hybridisation events detected according to the invention. It is expected that the skilled
30 person will realise that modifications may be made to improve the efficiency of the process. For example, improved washing steps, e.g. using a flow cell, would reduce

background noise and permit more concentrated solutions to be used, and hybridisation protocols could be adapted by varying the parameters of temperature, buffer, time etc.

CLAIMS

1. A device comprising an array of molecules capable of interrogation and immobilised on a solid surface, wherein the array has a surface density which allows the molecules to be individually resolved, and wherein each molecule is immobilised at one
5 or more points, by specific interaction with the surface, other than at that part of each molecule that can be interrogated.
2. A device according to claim 1, wherein at least 50% of the arrayed molecules are capable of being individually resolved.
3. A device according to claim 2, wherein at least 90% of the arrayed molecules are
10 capable of being individually resolved.
4. A device according to any preceding claim, wherein over 50% of the arrayed molecules are distinct.
5. A device according to any of claims 1 to 4, wherein the arrayed molecules are resolvable by optical microscopy.
- 15 6. A device according to any preceding claim, wherein the array has a surface density of one molecule per at least 10nm x 10nm.
7. A device according to claim 6, wherein the surface density is one molecule per at least 100nm x 100nm.
8. A device according to claim 6, wherein the surface density is one molecule per
20 at least 250nm x 250nm.
9. A device according to any preceding claim, wherein each molecule is conjugated to biotin, and is immobilised via interaction with streptavidin or avidin.
10. A device according to any preceding claim, wherein each molecule is immobilised via a microsphere.
- 25 11. A device according to claim 10, wherein the microspheres bear functional avidin or streptavidin and the solid surface has biotin bound thereto.
12. A device according to any of claims 1 to 8, wherein the molecules are immobilised via a covalent linkage.
13. A device according to any preceding claim, wherein each molecule is conjugated
30 to a fluorophore.

14. A device according to any preceding claim, wherein the molecules are polynucleotides immobilised to the solid support via the 5' terminus, the 3' terminus or via an internal nucleotide.
15. A device according to claim 14, wherein at least one arrayed polynucleotide has
5 a second polynucleotide hybridised thereto.
16. A device according to claim 14 or claim 15, wherein the arrayed polynucleotide is of known sequence.
17. Use of a device according to claim 14, for the capture of a second polynucleotide molecule capable of hybridising with the arrayed polynucleotide, comprising bringing into
10 contact with the device a sample containing or suspected of containing the second polynucleotide molecule, under hybridising conditions.
18. Use according to claim 17, wherein the sample is removed from contact with the device, thereby separating from the sample said second polynucleotide hybridised to an arrayed polynucleotide.
- 15 19. Use of a device according to any of claims 1 to 16 for monitoring an interaction with a single molecule, comprising resolving an arrayed molecule with an imaging device.
20. Use according to claim 19, wherein the arrayed molecule undergoes repeated interactions with each interaction being monitored.
21. A method for producing a device according to any of claims 1 to 16, comprising
20 immobilising a mixture of molecules onto a solid surface, wherein the molecules form an array having a surface density which allows the molecules to be individually resolved.
22. A method for forming a spatially addressable array, which comprises determining the sequences of a plurality of polynucleotide molecules immobilised on a device according to any of claims 1 to 16.
- 25 23. A method according to claim 22, further comprising the step of hybridising a polynucleotide molecule to its immobilised complement on the array.
24. A method according to claim 22, comprising the repeated steps of: reacting the immobilised polynucleotide with a primer, a polymerase and the different nucleotide triphosphates under conditions sufficient for the polymerase reaction to proceed, wherein
30 each nucleotide triphosphate is conjugated at its 3' position to a different label capable of being characterised optically, determining which label (and thus which nucleotide) has undergone the polymerisation reaction, and removing the label.

25. A method according to claim 24, wherein each label is a fluorophore.
26. A method for characterising a plurality of first molecules, comprising contacting, under suitable conditions, a spatially addressed array of second molecules with the first molecules, and detecting a binding event, wherein the array is as defined in any of claims 1 to 16.
27. A method according to claim 26, wherein the first molecules comprise a detectable tag.
28. A method according to claim 27, wherein the tag is a fluorophore.
29. A method according to claim 27, wherein the tag is a polynucleotide.
30. A method according to claim 29, wherein the polynucleotide sequence is determined.
31. A method according to claim 30, wherein the polynucleotide tag is removed after the sequence is determined.
32. A method for characterising an organism, comprising the steps of contacting a defined array of polynucleotide molecules immobilised on a solid support with a plurality of fragments of the organism's genomic DNA, under hybridising conditions, and detecting any hybridisation events, to obtain a distinct hybridisation pattern, wherein the array is as defined in any of claims 13 to 15.
33. A method according to claim 32, wherein the organism is human.
34. A method according to claim 32, wherein the organism is bacterial or viral.
35. A method according to any of claims 32 to 34, wherein the fragments of genomic DNA are detectably-labelled.
36. A method according to claim 35, wherein the label is a fluorophore.
37. A method according to any of claims 22 to 36 wherein the array comprises a solid support material having a plurality of cavities, each cavity comprising a polynucleotide molecule.

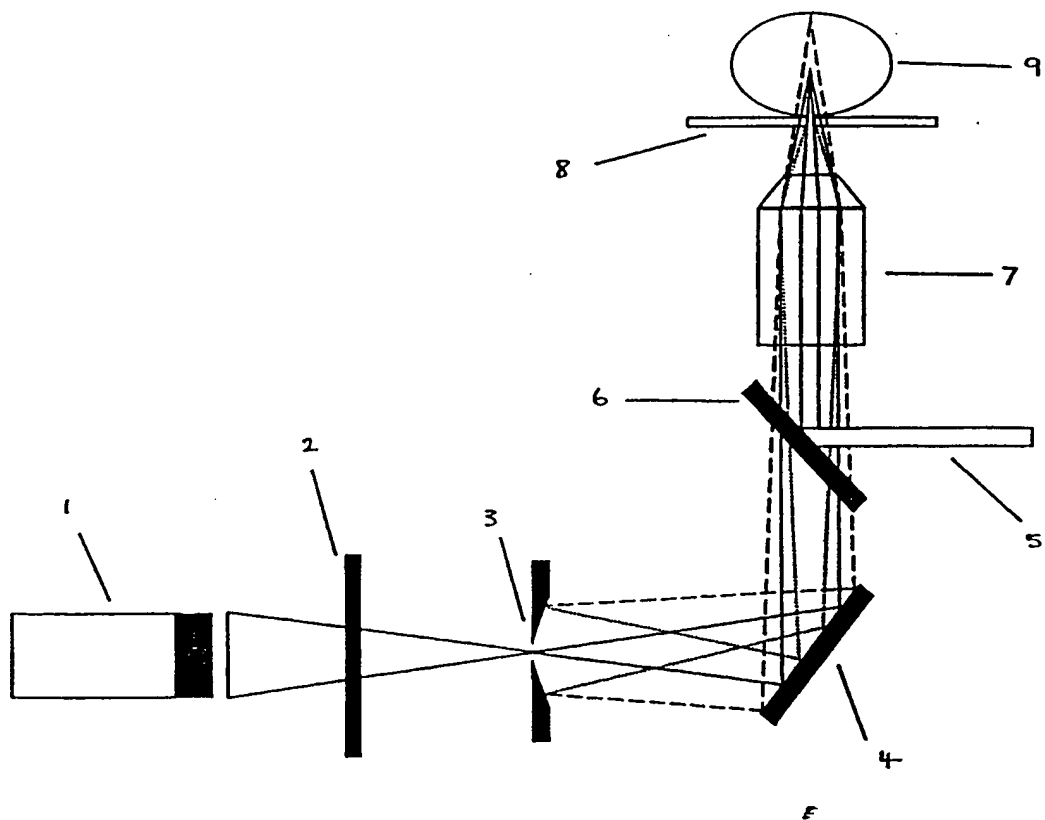


Figure 1

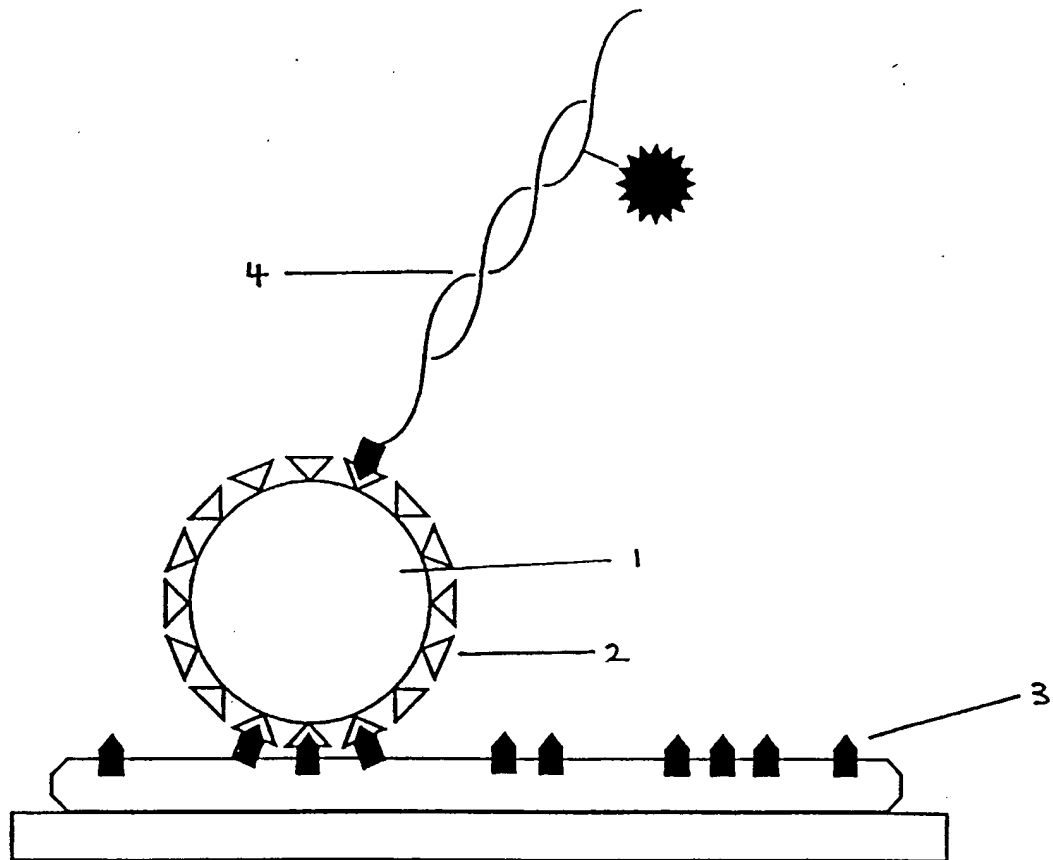


Figure 2

3/5

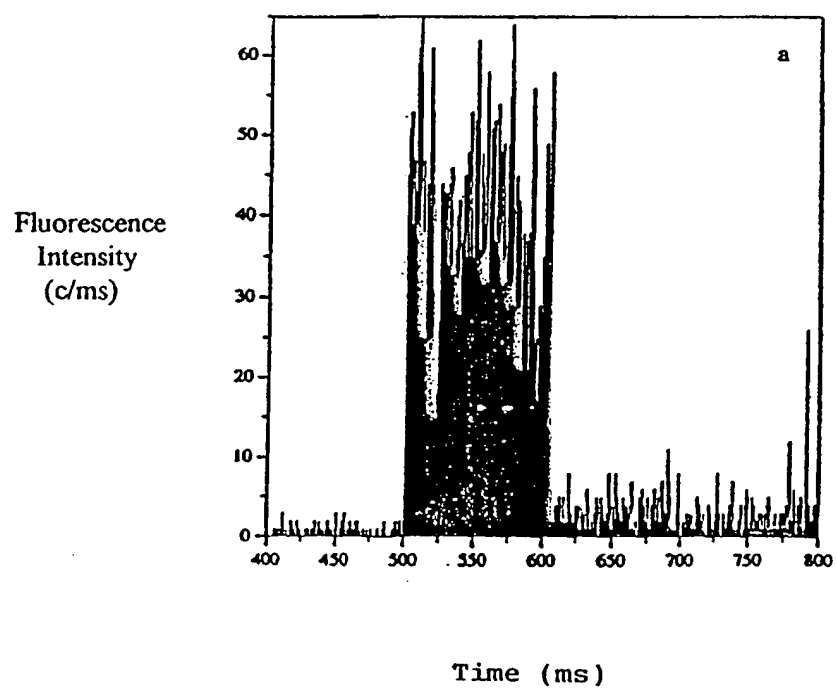


Figure 3

4/5

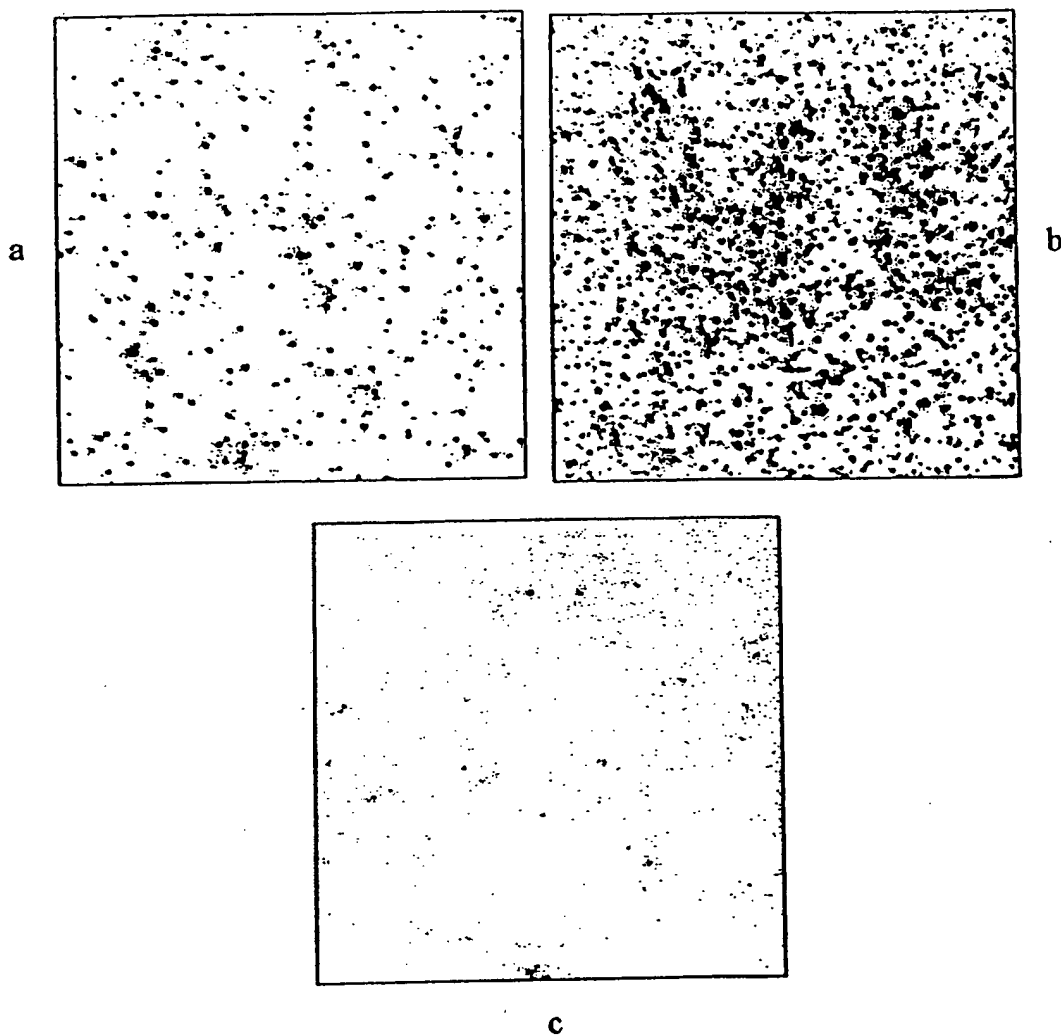


Figure 4

5/5

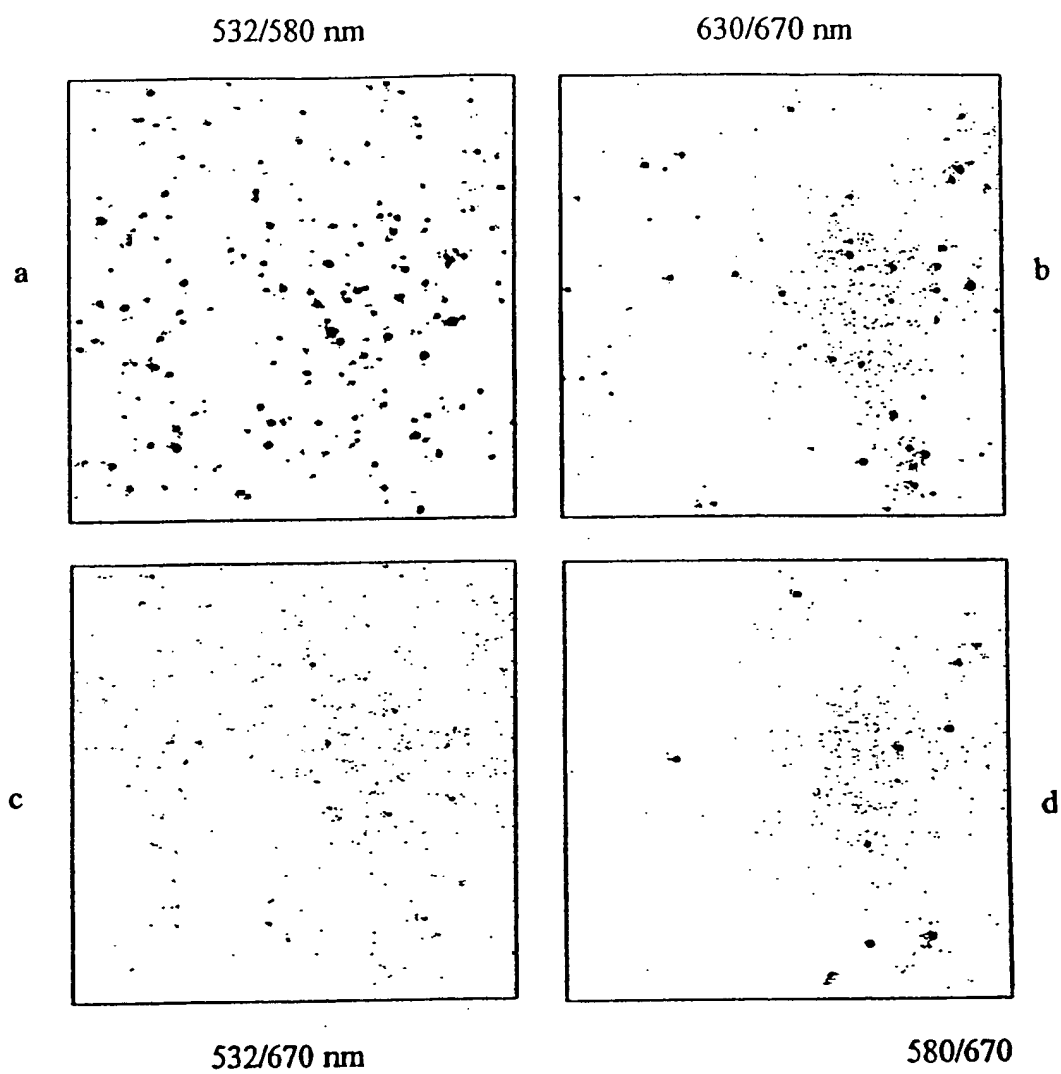


Figure 5

SEQUENCE LISTING

<110> Solexa Ltd

<120> ARRAYED BIOMOLECULES AND THEIR USE IN SEQUENCING

<130> REP05621WO

<140> n/a

<141> 1999-07-30

<160> 3

<170> PatentIn Ver. 2.1

<210> 1

<211> 13

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthetic

<220>

<221> misc_feature

<222> (1)..(13)

<223> Modified base. n = 5'-(propargylamino)uridine

<400> 1

tcgcagccgn cca

13

<210> 2

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthetic

<220>

<221> misc_feature

<222> (1)..(21)

<223> Modified base. n = 5-methyl cytosine with a TMR group coupled via a linker to the n4 position.

<400> 2

aaccctatgg acggctgcga n

21

<210> 3

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:synthetic

<220>

<221> misc_feature

<222> (1)..(21)

<223> Modified base. n= methyl cytosine.

<400> 3

ntcgagccg tccatagggt t

21

INTERNATIONAL SEARCH REPORT

Intern. Application No

PCT/GB 99/02487

A. CLASSIFICATION OF SUBJECT MATTER IPC 7 C12Q1/68 B01J19/00		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12Q B01J		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 20019 A (SEQUENOM, INC.) 14 May 1998 (1998-05-14)	1,9-12, 14,15, 17-19,21
A	the whole document	2-4,16, 22,23
A	WO 98 29736 A (GENOMETRIX INCORPORATED) 9 July 1998 (1998-07-09) abstract; claims; figures	1-37
X	US 5 314 829 A (L. STEPHEN COLES) 24 May 1994 (1994-05-24) abstract column 3, line 10 -column 4, line 35 figures	1,3-5, 11,15,18
A		2
-/--		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "Z" document member of the same patent family		
Date of the actual completion of the international search 11 January 2000		Date of mailing of the international search report 20/01/2000
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer Stevnsborg, N

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 99/02487

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DE 196 12 356 A (HANS-KNÖLL-INSTITUT FÜR NATURSTOFF-FORSCHUNG E.V.) 2 October 1997 (1997-10-02) the whole document ----	1-4, 9-18,21
A	EP 0 665 293 A (HAMAMATSU PHOTONICS K.K.) 2 August 1995 (1995-08-02) the whole document ----	1-4,9-37
A	US 5 780 231 A (SYDNEY BRENNER) 14 July 1998 (1998-07-14) abstract column 18, line 31 -column 24, line 3 figures ----	1-37
A	US 5 302 509 A (PETER C. CHEESEMAN) 12 April 1994 (1994-04-12) cited in the application the whole document ----	13,24, 25, 27-29, 35,36
A	EP 0 853 129 A (VYSIS, INC.) 15 July 1998 (1998-07-15) abstract; claims -----	1-37

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 99/02487

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9820019 A	14-05-1998	US 5900481 A	04-05-1999
		AU 5106998 A	29-05-1998
		AU 5247298 A	29-05-1998
		DE 19782097 T	14-10-1999
		EP 0954612 A	10-11-1999
		EP 0937097 A	25-08-1999
		NO 992167 A	05-07-1999
		NO 992168 A	06-07-1999
		WO 9820166 A	14-05-1998
		AU 5198098 A	29-05-1998
		EP 0937096 A	25-08-1999
		NO 992169 A	06-07-1999
		WO 9820020 A	14-05-1998
WO 9829736 A	09-07-1998	AU 6646398 A	31-07-1998
US 5314829 A	24-05-1994	NONE	
DE 19612356 A	02-10-1997	NONE	
EP 665293 A	02-08-1995	JP 7203998 A	08-08-1995
US 5780231 A	14-07-1998	US 5763175 A	09-06-1998
		AU 1161997 A	22-09-1997
		EP 0840803 A	13-05-1998
		WO 9732999 A	12-09-1997
		US 5962228 A	05-10-1999
US 5302509 A	12-04-1994	NONE	
EP 853129 A	15-07-1998	US 5837466 A	17-11-1998
		JP 10293128 A	04-11-1998

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☒ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.